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An Association Between *RRM1* Haplotype and Gemcitabine-Induced Neutropenia in Breast Cancer Patients

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ABSTRACT

Purpose. We examined the pattern of single-nucleotide polymorphisms (SNPs) of gemcitabine metabolism-related and target genes in breast cancer patients and evaluated their association with drug response or toxicity.

Patients and Methods. SNPs in deoxycytidine kinase (dCK), deoxycytidine monophosphate deaminase (DCTD), and ribonucleotide reductase M1 polypeptide (RRM1) were analyzed with genomic DNA of 10 breast cancer cell lines, 74 peripheral blood mononuclear cell (PBMC) samples from advanced breast cancer patients treated with gemcitabine, and 56 PBMC samples from healthy volunteers.

Results. The incidences of SNPs of breast cancer pa-

tients were 1.4% in dCK (626 A>G), 10.8% in DCTD (315 T>C), 40.5% in the first RRM1 (1082 C>A), 44.6% in the second RRM1 (2455 A>G), 44.6% in the third RRM1 (2464 G>A), and 23% in two RRM1 sites (2455 A>G and 2464 G>A) that were similar to those of the normal control group. We found a double SNP of RRM1 (2455 A>G and 2464 G>A) to be the novel haplotype that was associated with a lower frequency of chemotherapy-induced toxicity, such as neutropenia ($p < .01$) and G-CSF requirement ($p < .005$).

Conclusion. RRM1 haplotype showed an association with susceptibility to gemcitabine monotherapy in breast cancer patients. *The Oncologist* 2007;12:622–630

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Gemcitabine (2′2-difluorodeoxycytidine [dFdC]) is a specific analogue of the native pyrimidine nucleotide deoxycytidine. It is a novel anticancer agent that has significant activity in carcinomas of the ovary, lung, pancreas, and breast [1]. Gemcitabine has a complex metabolic pathway for cytotoxicity [2, 3]. It is transported into the cell by nucleoside transporters, phosphorylated by deoxycytidine kinase (dCK) to its active monophosphate form [4], further phosphorylated to the triphosphate form, and then incorpo-

rated into the DNA. Therefore, dCK plays a key role in the activation of gemcitabine and its activity correlates with drug sensitivity [5–9]. Gemcitabine is inactivated by deoxycytidine monophosphate deaminase (DCTD) into its inactive form of difluorodeoxyuridine (dFdU). Ribonucleotide reductase (RR) is the rate-limiting step in DNA synthesis. It is the only known enzyme that converts ribonucleotides to deoxyribonucleoside for DNA polymerization and repair [10]. RR is a holoenzyme consisting of dimerized RR subunit 1 and 2 (RRM1, RRM2). RRM1 has

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Table 1. Patient characteristics

	Patients n (%)	Healthy volunteers n
Total n of patients	74	56
Evaluated n of patients	71	
Median age (years)	50	40
Range	(31–70)	(20–64)
Performance status score		
0–1	46 (62.2)	
2	28 (37.8)	
Menopausal status		
Premenopausal	50 (67.6)	
Postmenopausal	24 (32.4)	
Histology		
Ductal carcinoma	72 (97.3)	
Lobular carcinoma	2 (2.7)	
Hormone receptor status		
ER ⁺ PgR ⁺	24 (32.4)	
ER ⁺ PgR [−]	23 (31.1)	
ER [−] PgR ⁺	9 (12.2)	
ER [−] PgR [−]	16 (21.6)	
Unknown	2 (2.7)	
Abbreviations: ER, estrogen receptor; PgR, progesterone receptor.		

been shown to function with the p53-regulated RRM2 homologue p53R2, which is important in DNA repair secondary to genotoxic stress [11]. In in vitro studies, increased RRM1 expression and activity has been shown to be a marker for gemcitabine resistance [12–14].

In gemcitabine metabolism, where 13 genes are involved, the first step in phosphorylation is catalyzed by dCK, which is the rate-limiting step for further phosphorylation to active metabolites, and thus is essential for the activation of gemcitabine. Alternatively, gemcitabine is inactivated by DCTD into its inactive form. RRM1 is the rate-limiting step of DNA synthesis and is inhibited by diphosphorylated gemcitabine (dFdCDP). dCK deficiency, increased DCTD, and increased RR activity are the main mechanisms of gemcitabine resistance. Therefore, we chose these three rate-limiting steps in our study focusing on the gemcitabine metabolic pathway (DCTD, dCK) or target molecule of gemcitabine (RRM1) as an association marker of clinical outcome (response, toxicity) of gemcitabine monotherapy.

For single-nucleotide polymorphism (SNP) site selection, we tried to select nonsynonymous SNPs in the functional and conserved region of the genes among known

SNPs when we designed this study. Therefore, we tried to choose SNPs in the conserved coding region without any known function of the SNP. With these criteria, we chose two cSNP sites from *DCTD* (site 255 and 351) and two cSNP sites from *dCK* (site 626 and 753) with no known function. Among nine SNP sites in *RRM1*, we finally chose three nonsynonymous cSNP sites (1082, 2455, 2464) located in the LD block and located in the protein domain and conserved region of *RRM1*.

With the development of a rapid and practical method of genetic polymorphism analysis, we detected polymorphisms of *dCK*, *DCTD*, and *RRM1* in advanced breast cancer patients who received gemcitabine monotherapy. And we performed an association study to test the relationship between clinical phenotypes and those genetic polymorphisms.

PATIENTS AND METHODS

Patients and Healthy Volunteers

Seventy-four patients treated at Yonsei Cancer Center for advanced breast cancer were enrolled in the study. Patients eligible for this study had histologically confirmed breast cancer with documented progression after prior use of anthracyclines and taxanes. All patients were required to have a measurable lesion, to be 75 years of age or younger, to have an Eastern Cooperative Oncology Group (ECOG) performance status score of ≤ 2 , to have an expected survival time of >12 weeks, and to have adequate bone marrow and renal and hepatic functions (hemoglobin >10.0 g/dl, leukocytes $>3,000/\text{mm}^3$, platelets $>100,000/\text{mm}^3$, total bilirubin and serum creatinine $<1.5\times$ the upper normal limit and aspartate aminotransferase and alanine aminotransferase $<2\times$ the upper normal limit). Prior therapy had to have been completed at least 4 weeks before study entry with full resolution of toxicities. Informed consent was obtained according to the institutional regulations. The study protocol was approved by the institutional review board. As a control, 56 healthy unrelated female blood donors were included in the study. Patient characteristics are summarized in Table 1.

Treatment Plan

Chemotherapy consisted of a single dose of gemcitabine, 850 mg/m^2 , on days 1, 8, and 15 every 28 days. There was no modification of gemcitabine dose during the whole treatment. If the patient had grade III leukopenia, the patient received G-CSF support ($150 \mu\text{g/m}^2$) and the treatment was delayed until recovery on a weekly base. No prophylactic G-CSF was administered.

Assessment of Response and Toxicity

Antitumor activity was evaluated every three courses. Tumor response was measured according to World Health Organization (WHO) criteria. In every course, toxicity was evaluated by National Cancer Institute Common Toxicity Criteria version 3.0.

Cell Lines and Cell Culture

Six human breast cancer cell lines (MCF/ADR, MDA-MB-231, MDA-MB-435, MCF-7, T47D, and SK-BR-3) were obtained from the American Type Culture Collection (Rockville, MD). We also added another four cell lines (YCC-B1, YCC-B2, YCC-B3, and YCC-B5) that had been established from Korean breast cancer patients (Cancer Metastasis Research Center, Seoul, Korea). The cells were cultured and maintained in minimal essential medium supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY), 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin (GIBCO) in a humidified 37°C incubator with 5% CO₂.

Drug Sensitivity Test by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetra Zoliumbromide (MTT) Assay

Cells (1×10^4) were inoculated into each well of a 96-well plate with various concentrations of gemcitabine. The plates were incubated for 72 hours and then 5 μ g of MTT was added to each well. After dissolving formazan, the plates were read immediately at 570 nm using a model 550 Micro Plate Reader (Bio-Rad, Hercules, CA). The assays were repeated three times.

Genomic DNA Extraction from Cell Lines and Lymphocytes

The cultured cells were resuspended with DNA lysis buffer (10 mM Tris-HCl [pH 7.6], 10 mM EDTA, 50 mM NaCl, 0.2% SDS, 20 mg/ml proteinase K) and incubated overnight at 42°C. Using phenol:chloroform:isopropanol alcohol (GIBCO), we extracted genomic DNA and the pellet was washed with 70% ethanol. The final concentration was determined with Gene Quant RNA/DNA Calculator (Amersham Pharmacia Biotech USA).

Peripheral blood lymphocytes were collected in heparinized vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). Lymphocytes were isolated using Ficoll-Paque (Pharmacia, Uppsala, Sweden) following the manufacturer's instructions. Genomic DNA from lymphocytes was isolated with the LaboPass™ Blood kit (Genotein Biotech, Seoul, Korea). Extracted DNA was stored at -70°C.

SNP Target Determinations

Target SNPs were determined using available SNP public databases. The GeneCards (<http://bioinformatics.weizmann.ac.il/cards>) and OMIM (<http://www.ncbi.nlm.nih.gov/Omim>) databases were used to gain information about the genes in the gemcitabine metabolic pathway. Database queries for SNPs in the gemcitabine metabolic pathway were conducted in HOWDY (<http://gdb.jst.go.jp/HOWDY/>), which includes dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>) and JSNP (<http://snp.ims.utokyo.ac.jp/>) [2].

Polymerase Chain Reaction

The primer sequences used were as follows: *DCTD* exon 3 (forward, 5'-CATCAGCAATGAGCTACTGA; reverse, 5'-TGCAACC AAAGTTTTTCTTT), *dCK* exon 4 (forward, 5'-CCACTGGATTAGGAGAATG; reverse, 5'-GTGAAACACATTTTATTGGG), *dCK* exon 5 (forward, 5'-AAAAGAAAATTTTGATG GCA; reverse, 5'-ACTTCAGTGCCTATGCAGG), *RRM1* exon 9 (forward, 5'-TTGATTTTAT TTGGGCATTT; reverse, 5'-CAATTCAG GAGCATACCTT), and *RRM1* exon 19 (forward, 5'-TT CCTTGTAGGGTTTGAAGA; reverse, 5'-AGGATCCACACATCAGACAT). The conditions of the polymerase chain reaction (PCR) amplification were as follows: 95°C for 5 minutes followed by 94°C for 30 seconds, 57°C for 20 seconds, 72°C for 30 seconds, and 72°C for 2 minutes repeated for 30 cycles.

DNA Sequencing

Direct sequencing of each PCR product was carried out using CEQ™ 8000 genetic analysis system (Beckman Coulter, Inc., Fullerton, CA). The reaction mixture contained 25–100 fmol of purified PCR products, 1.6 pmol/ μ l of either the sense or antisense oligonucleotides (same as PCR primer) and DTCS premix (10 \times sequencing buffer, dNTP mix, ddUTP/ddCTP/ddATP/ddGTP dye terminator, polymerase enzyme). Each cycle of the sequencing reaction consisted of 96°C for 20 seconds, 50°C for 20 seconds, and 60°C for 4 minutes. The PCR products were dissolved in Sample Loading Solution (CEQ Dye Terminator cycle sequencing kit, Beckman Coulter, Inc.). After loading on the CEQ™ 8000 genetic analysis system, fluorescence was detected. Sequence variances were checked by comparing them with reference sequences obtained from GenBank (*DCTD*, NM_001921.1; *dCK*, NM_000788; *RRM1*, NM_001033.2).

Real-Time PCR Assay

RNA extraction was performed using TRIzol Reagent (Invitrogen Corporation, Carlsbad, CA). The primer

Table 2. Toxicity of chemotherapy by World Health Organization grade

Toxicity	World Health Organization grade				
	0	1	2	3	4
Number of patients (%)					
Hematologic toxicity					
Leukopenia	11 (14.9)	19 (25.7)	28 (37.8)	16 (21.6)	0 (0.0)
Neutropenia	14 (18.9)	15 (20.3)	21 (28.4)	22 (29.7)	2 (2.7)
Anemia	1 (1.4)	29 (39.2)	38 (51.4)	6 (8.1)	0 (0.0)
Thrombocytopenia	40 (54.1)	14 (18.9)	16 (21.6)	3 (4.1)	1 (1.4)
Nonhematologic toxicity					
Diarrhea	21 (28.4)	44 (59.5)	9 (12.2)	0 (0.0)	0 (0.0)

sequences used were as follows: *RRM1* (forward, 5'-ATCAGGACTGGTCTTTGATG-3'; reverse, 5'-TGA GACTCAATGATGGCATA-3'), *β -actin* (forward, 5'-GGGAATTCAAACTGGA ACGGTGAAG G-3'; reverse, 5'-GGAAGCTTATCAAAGTCCTCGGCCAC A-3'). PCR was performed at 95°C for 15 minutes in order to activate the HotstarTaq DNA polymerase, and then for 35 cycles of amplification at: 95°C for 20 seconds, 50°C for 30 seconds, and 72°C for 45 seconds. The amplified fluorescence signal in each specimen was measured at the last extension step of each cycle. In order to quantify each gene, we used 10-fold serially diluted human genomic DNA (Promega, Madison, WI). The standard curve was drawn by plotting the measured threshold cycle versus the arbitrary unit of the copies per reaction based on the serially diluted genomic DNA of *β -actin*. The threshold cycle (C_t) values were determined as the cycle number at which the fluorescence exceeded the threshold value. In the negative control, there was no fluorescent signal even after the cycle number was increased more than 35-fold.

Western Blotting

Cells were resuspended in 50 μ l of lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM CaCl_2 , 1 mM MgCl_2 , and protease inhibitor mixture). The amount of protein was quantitated using the Bradford Protein Assay (Bio-Rad Laboratories, Hercules, CA). The antibodies used were mouse monoclonal anti-RRM1 (1:500; Chemicon International, Temecula, CA), antimouse IgG HPL whole antibody (1:2000; Amersham Pharmacia Biotech, Little Chalfont, UK), and anti- *β -actin* (Abcam Ltd., Cambridge, UK). After adding the ECL blotting reagents (Amersham Pharmacia Biotech), the membrane was exposed to high performance autoradiography film (Amersham Pharmacia Biotech).

Statistical Analysis

Clinical data analysis was performed using the SPSS 10.0 program (SPSS Inc., Chicago, IL). Time-dependent variables were estimated with a log-rank test using the Kaplan–Meier method. We used the χ^2 test to correlate the SNPs with toxicities. Allele or genotype frequency differences between the patient and control populations were determined based on Hardy–Weinberg equilibrium using SNP analyzer software (http://www.istech21.com/bionics/consulting_6.htm). Haplotypes were constructed from ge-

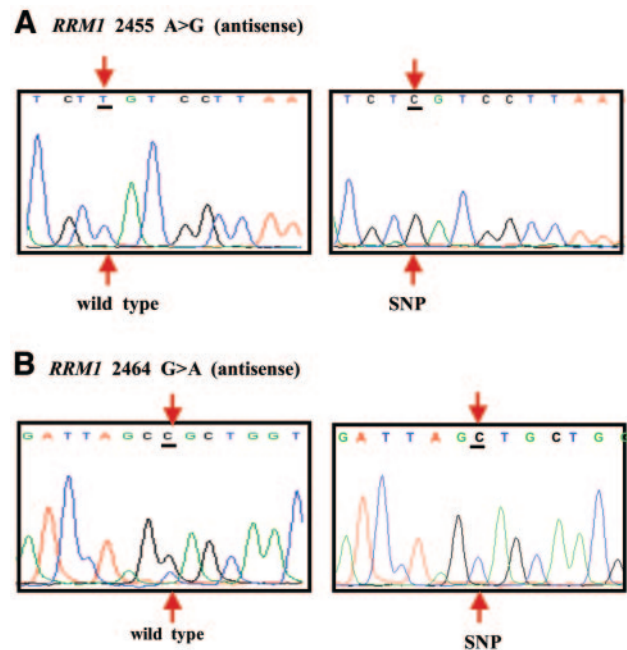


Figure 1. Electropherograms of two nonsynonymous SNPs and their flanking sequences in *RRM1*. Arrows indicate the polymorphic and homozygous positions, and the homozygous nucleotides are underlined.

Abbreviations: RRM1, ribonucleotide reductase M1 polypeptide; SNP, single-nucleotide polymorphism.

Table 3. Frequency of each SNP in breast cancer patients and healthy volunteers

Gene	SNP site	SNP type	Amino acid change	Patients (n = 74)		Healthy volunteers (n = 56)	
				Wild type (%)	Homozygous (%)	Wild type (%)	Homozygous (%)
<i>DCTD</i>	Exon 4	255 G>C	Ala/Ala	74 (100)	0 (0.0)	56 (100)	0 (0.0)
		315 T>C	Val/Val	66 (89.1)	8 (10.8)	52 (92.5)	4 (7.1)
<i>dCK</i>	Exon 4	626 A>G	Gln/Arg	73 (98.6)	1 (1.4)	56 (100)	0 (0.0)
	Exon 5	753 A>G	Gln/Gln	74 (100)	0 (0.0)	56 (100)	0 (0.0)
<i>RRM1</i>	Exon 9	1082 C>A	Arg/Arg	65 (87.7)	9 (12.1)	28 (50.0)	28 (50.0)
	Exon 19	2455 A>G	Thr/Thr	44 (59.5)	30 (40.5)	17 (30.4)	39 (69.6)
		2464 G>A	Ala/Ala	41 (55.4)	33 (44.6)	20 (35.7)	36 (64.3)
		2455 A>G + 2464 G>A		57 (77.0)	17 (23.0)	26 (46.4)	30 (53.6)

Abbreviations: *DCTD*, deoxycytidine monophosphate deaminase; *dCK*, deoxycytidine kinase; *RRM1*, ribonucleotide reductase M1 polypeptide; SNP, single-nucleotide polymorphism.

notype data using SNP analyzer software (http://www.istech21.com/bionics/consulting_6.htm). The results were considered significant when two-sided *p*-values were <.05.

RESULTS

Patient Characteristics

Baseline characteristics for the 74 patients are shown in Table 1. Among 71 evaluable patients, one complete response (1.4%), 13 partial responses (18.3%), and 26 cases of stable disease (36.6%) were documented. The overall response rate was 19.7% and the disease control rate was 56.3%. The most common toxicity was neutropenia, and 32.4% of patients reported grade III–IV neutropenia. Only 5.5% of the patients showed grade III–IV thrombocytopenia. Nonhematologic toxicity was mild and only grade II diarrhea was reported in 12.2% of the patients (Table 2).

Frequencies of SNPs

In breast cancer patients, the incidences of SNPs were 1.4% in *dCK* (626 A>G), 10.8% in *DCTD* (315 T>C), 12.1% in the first *RRM1* (1082 C>A), 40.5% in the second *RRM1* (2455 A>G), 44.6% in the third *RRM1* (2464 G>A), and 23.0% in the two *RRM1* SNP sites (2455 A>G and 2464 G>A) (Fig. 1 and Table 3). In healthy volunteers, SNPs were not found in *dCK*, and the incidences of SNPs were 7.1% in *DCTD*, 50.0% in the second *RRM1*, 64.3% in the third *RRM1*, and 53.6% in the two *RRM1* sites (2455 A>G and 2464 G>A). When allele distributions of the three genes were evaluated by the Hardy–Weinberg equation, there were no differences in SNP frequencies between healthy volunteers and breast cancer patients.

Association Between *RRM1* SNP and Clinical Phenotype

The associations between *RRM1* SNP and treatment response and toxicity were analyzed in breast cancer patients. There were no differences in toxicity between patients having any type of single SNP and patients having a wild-type allele. However, in patients with double SNPs (2455 A>G and 2464 G>A) in *RRM1*, less toxicity, such as less neutropenia (*p* < .01), less leukopenia (*p* < .004), a shorter treatment delay (*p* < .002), and a lower G-CSF requirement (*p* < .004) were observed when compared with wild-type or single SNP patients (Fig. 2A–E). However, we could not observe any significant association between SNP and tumor response after gemcitabine monotherapy (data not shown).

Association Between *RRM1* Haplotype and Gemcitabine Toxicity

With three *RRM1* SNPs, we constructed six haplotypes (Table 4). A strong association was found between the lower frequency of neutropenia and a haplotype containing two SNPs (2455 A>G and 2464 G>A). No association was observed between clinical response and *RRM1* haplotype (wild type, 26%; one SNP, 15%; double SNP, 18% response rate) (Table 5). There was a tendency toward a poor progression-free survival time in patients having the two SNPs (2455 A>G and 2464 G>A) when compared with wild-type or single SNP patients. In addition, poor overall survival was found in patients having the two SNPs when compared with wild-type or single SNP patients (Fig. 3). The median times to progression of the patients with wild type or single SNP and those with two SNPs were 17 and 12 weeks, respectively. The median overall survival times of

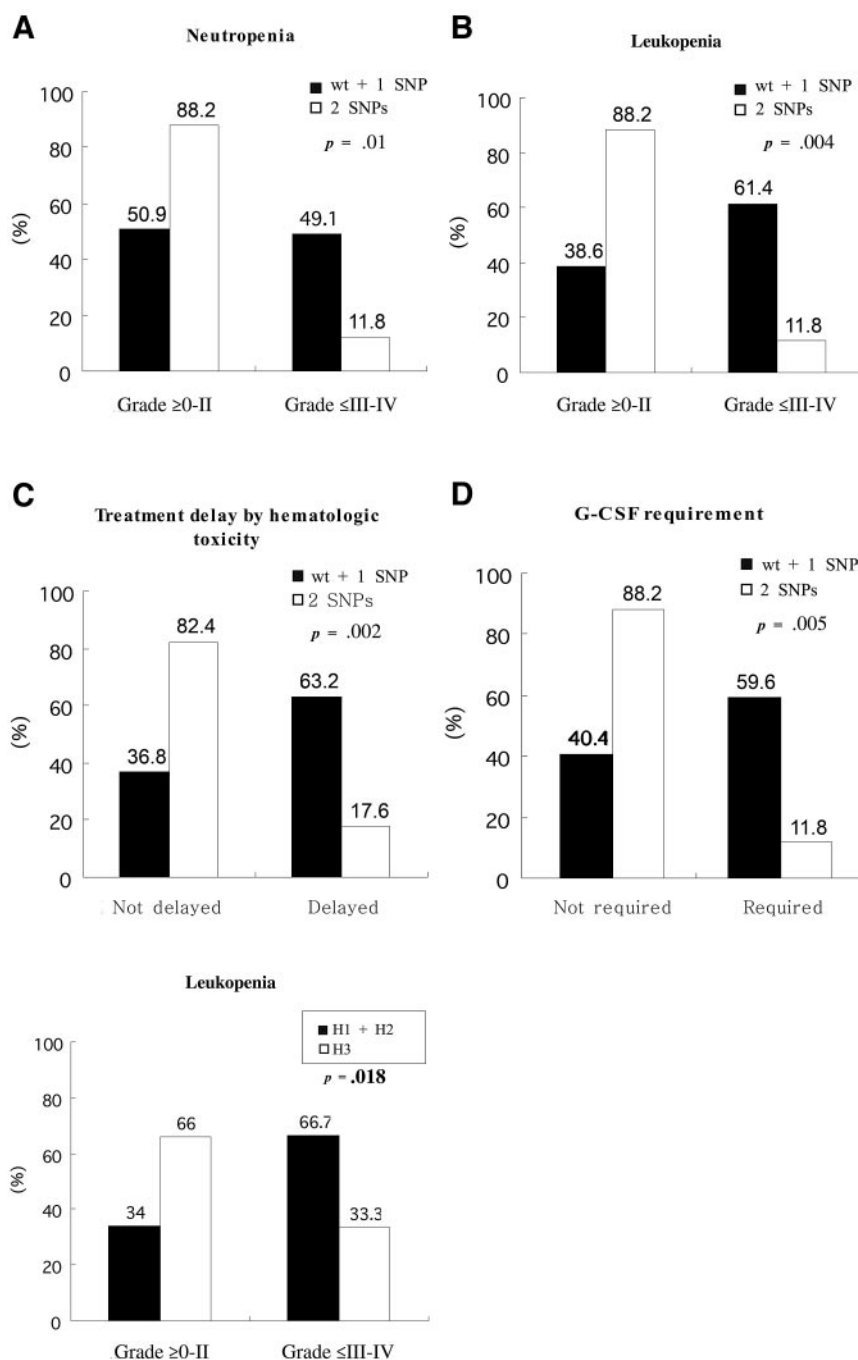


Figure 2. Comparison of gemcitabine toxicity based on *RRM1* SNPs. (A): Neutropenia. (B): Leukopenia. (C): Treatment delay. (D): G-CSF requirement. (E): Comparison of leukopenia based on *RRM1* haplotype.

Abbreviations: *RRM1*, ribonucleotide reductase M1 polypeptide; SNP, single-nucleotide polymorphism; wt, wild type.

the patients with wild type or single SNP and those with two SNPs were 60 and 43 weeks, respectively.

In Vitro Association Between *RRM1* SNP and Chemosensitivity

We selected ten breast cancer cell lines to perform an association study between type of *RRM1* SNP and in vitro cy-

totoxicity to gemcitabine. In breast cancer cell lines, as in patients, *RRM1* SNPs appeared more frequently than *dCK* and *DCTD* SNPs. The incidences of SNPs were 50.0% in the first *RRM1* (1082 C>A), 70.0% in the second *RRM1* (2455 A>G), 70.0% in the third *RRM1* (2464 G>A), and 60.0% in the two *RRM1* SNP sites (2455 A>G and 2464 G>A).

Table 4. *RRM1* haplotypes

	Haplotype	Patients, frequency (%)	Cell line, frequency (%)
<i>H1</i>	C A G	26 (35.1)	2 (20.0)
<i>H2</i>	C A A	14 (18.9)	1 (10.0)
	C G G	10 (13.5)	1 (10.0)
	A A G	3 (4.1)	0 (0.0)
<i>H3</i>	C G A	16 (21.6)	1 (10.0)
	A G A	2 (2.7)	5 (50.0)
	A G G	3 (4.1)	0 (0.0)
	Total	74	10

H1, wild type; *H2*, single SNP site (1082 C>A, 2455 A>G, 2464 G>A); *H3*, double SNP (2455 A>G and 2464 G>A).
Abbreviation: SNP, single-nucleotide polymorphism.

There were no differences in gemcitabine sensitivity between cell lines with single SNP and wild-type genotypes (data not shown). Also, we observed that six cell lines with two *RRM1* SNPs (2455A>G and 2464 G>A) showed a similar sensitivity to gemcitabine when compared with the cell lines with wild type or one SNP (Fig. 4A). Cell lines with *RRM1* SNPs showed similar RNA and protein expression to those of wild-type cell lines (Fig. 4B).

DISCUSSION

Pharmacogenetics aims to identify genetic polymorphisms that govern an individual's response to specific drugs [15]. Clinical observation of an inherited difference in drug effect was first documented in the 1950s giving rise to the field of pharmacogenetics later. One of the common variations in the human genome is SNP. More than 1.4 million SNPs were identified in the initial sequencing of the human genome, with over 60,000 of these in the coding region of genes. Some of these SNPs have been shown to be associated with substantial changes in metabolism or in the effect of anticancer agents, and some SNPs are now being applied to predict clinical outcome [16–18].

In patients with double SNPs (2455 A>G and 2464

G>A) in *RRM1*, less toxicity was observed, suggesting an association with treatment susceptibility. In contrast, we could not observe any significant association between SNP and tumor response. To date, few studies have addressed the issue of the impact of SNP on chemotherapy efficacy. The 5' tandem repeat polymorphism on the thymidylate synthase gene has been identified as a predictor of clinical outcome for 5-fluorouracil-based chemotherapy in colorectal cancer and acute lymphoblastic leukemia [19]. Because a double SNP was found to be associated with gemcitabine toxicity, we aimed to assess the potential role of specific haplotypes in influencing gemcitabine susceptibility. A strong association was observed between neutropenia and the *RRM1* haplotype containing two SNPs (2455 A>G and 2464 G>A). No association was observed between clinical response and *RRM1* haplotype, but there was a tendency toward poor progressive-free survival in patients having the haplotype with double SNPs when compared with wild-type or single SNP patients. We suggest that patients with the *RRM1* haplotype 2455 A>G and 2464 G>A are genetically more resistant to gemcitabine (relatively poor progression-free survival) and less susceptible to gemcitabine (less general toxicity) than wild-type or single SNP *RRM1* patients.

It is difficult to draw a definite conclusion from an association study between the SNPs of genomic DNA and clinical phenotypes. Our hypothesis was that patients with sequence variations might show different responses or toxicities from those of patients with wild-type alleles. A silent mutation that does not change the amino acid sequence can alter protein function. In contrast, a wobble protein, as in our cases, can show differences in clinical phenotypes. The selected SNP sites in our study are located at the end of mRNA and they might be implicated in nucleotide transport or metabolism. Alternatively, the anticodon or other molecules may be indirectly influenced during transcription or translation. Similarly, the molecular mechanisms of transcription enhancement by tandem repeats in the excision-repair cross-complementing 1 gene remain largely unknown [19].

Table 5. Comparison of tumor response based on *RRM1* haplotype

	Haplotype	CR/PR (%)	SD (%)	PD (%)	Total (n = 71)
Wild type	<i>H1</i>	6 (26.0)	9 (39.0)	8 (35.0)	23
1 SNP	<i>H2</i>	4 (15.0)	9 (35.0)	13 (50.0)	26
2/3 SNP	<i>H3</i>	4 (18.0)	8 (36.0)	10 (45.0)	22

Abbreviations: CR, complete response; PD, progressive disease; PR, partial response; SD, stable disease; SNP, single-nucleotide polymorphism.

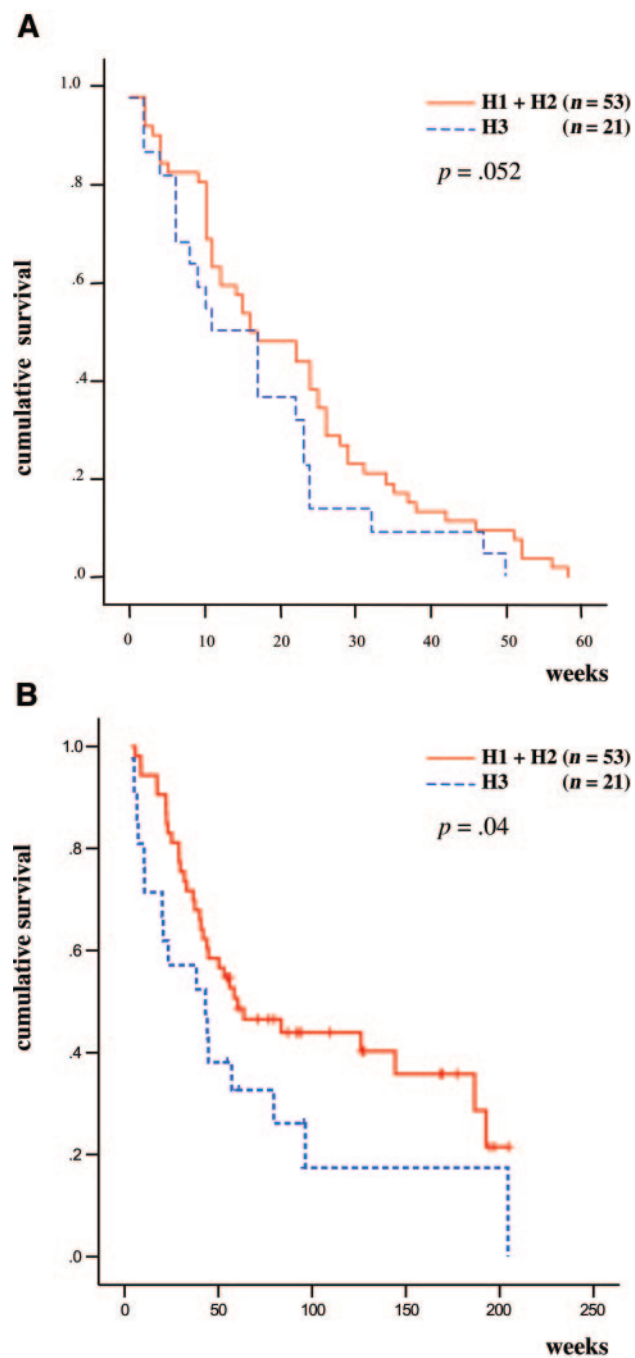


Figure 3. Comparison of survival according to *RRM1* haplotype. (A): Progression-free survival. (B): Overall survival. *H1*, wild-type allele; *H2*, single SNP; *H3*, double SNP.

Abbreviations: RRM1, ribonucleotide reductase M1 polypeptide; SNP, single-nucleotide polymorphism.

RR is usually overexpressed in cancer cells, and therefore RR inhibitors might be useful as anticancer drugs, like gemcitabine [20]. It has been reported that the increase in mRNA level of RRM1 results in drug resistance [12]. With nonsynonymous SNP sites in *RRM1* gene, we found similar chemosensitivity, mRNA, and protein level as with the

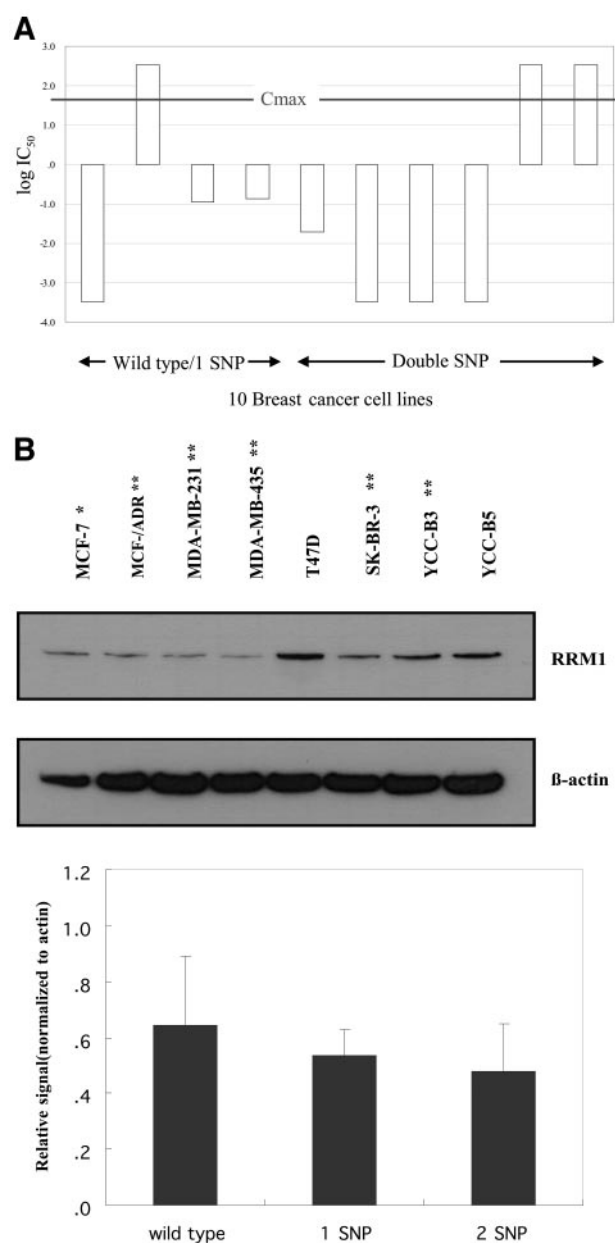


Figure 4. In vitro study of *RRM1* SNP and chemosensitivity. (A): Chemosensitivity (IC_{50}) of ten breast cancer cell lines. (B): Expression levels of RRM1 in cancer cell lines. *Single SNP, **double SNP.

Abbreviations: IC_{50} , 50% inhibitory concentration; RRM1, ribonucleotide reductase M1 polypeptide; SNP, single-nucleotide polymorphism.

wild type. In contrast, breast cancer patients having double SNPs (2455 A>G and 2464 G>A) showed resistance to gemcitabine. Possible explanations for this gap between the in vitro and in vivo results are: first, double SNPs can cause a different phenotype than the single SNP in *RRM1* during chronic exposure to chemotherapy in patients, and second, with two SNPs, a difference in the three-dimensional structure might be induced in patients without any change at the

transcription and translation level of RR. One retrospective study indicated that RRM1 levels might influence both the response and survival of non-small cell lung cancer patients to gemcitabine–cisplatin combination therapy [21]. RRM1 is suggested to act as a “molecular sink” for gemcitabine, whereby the drug binds irreversibly to subunit RRM1 and inactivates it. Therefore, our hypothesis is that the altered sequence of *RRM1* might induce a structural change in RRM1 that could easily bind to gemcitabine and thus induce drug resistance. Under this hypothesis, we chose cSNP in a nonsynonymous form located within the functional and conserved region of the gene. For the confirmation of this hypothesis, an RRM1 simulation study with a three-dimensional structure and biochemical binding assay to gemcitabine is required in the future. Also, further molecular study is needed for understanding the mechanism of gemcitabine resistance with double SNPs of *RRM1*.

Although we cannot definitely predict drug toxicity or efficacy with the cSNPs of *RRM1*, the haplotype with dou-

ble SNPs (2455 A>G and 2464 G>A) showed a high association with gemcitabine susceptibility, suggesting this haplotype as a potential association marker for gemcitabine compliance. A prospective comparison study using *RRM1* SNP as a biomarker for gemcitabine toxicity, using it as a randomization factor, is warranted in the future. With this prospective confirmation, *RRM1* SNP can be used as a biomarker for the prediction of toxicity or prognosis.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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